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Selective fluorescence detection of Cu^{2+} in aqueous solution and living cells

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ABSTRACT

A rhodamine B semicarbazide **3** was synthesized by the reaction of rhodamine B acid chloride **2** with hydrazine carboxamide hydrochloride under reflux with triethyl amine in acetonitrile. It was used as selective fluorescent and colorimetric sensor for visual detection of Cu^{2+} over competitive ions (Fe^{3+}, Fe^{2+}, Cr^{3+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Ag^{2+}, Mn^{2+}, Sr^{2+}, Cs^{2+}, Na^{+}, K^{+}, Li^{+}) in aqueous methanol (1:1, v/v), exhibiting a fast response time, less than few second and a detection limit of 1.6×10^{-7} mol/L at neutral pH. The proposed sensing system can be successfully applicable for determination of Cu^{2+} in waste water samples showing turn on fluorescence response and for further monitoring of intracellular Cu^{2+} levels in living cells with high sensitivity and selectivity at micro molar level concentrations using confocal fluorescence spectroscopy. The synthesis of probe **3** was confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometric analysis.

1. Introduction

Various toxic chemicals produced during industrial and agricultural activities, contaminating the aquatic ecosystems, is a global environmental problem. Elevated level of heavy metals causes harmful effects on the health of aquatic organisms and their consumers [1]. Copper, an essential trace metal for life, is present in natural water, sediments and in the other medium including air and soil. It is abundant in factory effluents, *i.e.*, factories related to manufacturing of electronic goods, fertilizers, fungicides and in plating byproducts. Sources of copper contamination include mining and smelting, urban, industrial and agricultural wastes, and the use of agrochemicals. Copper involved in metabolic processes that depend on redox reactions and the body can regulate its level haemostatically. Although large, acute doses can have harmful effects, even fatal causing hemolysis, jaundice and death. In addition, copper causes damage to a variety of aquatic fauna and possesses carcinogenic effect in human [2]. Higher concentration of copper in an aquatic ecosystem lead to deleterious effect on human health specially in infants and young children through ingestion and metal contaminated water causing number of medical disorders due to increased production of free radicals in the body [3], teratogenicity [4] and chromosomal aberrations [5]. Therefore, the protection of aquatic habitat from damaging, due to such contaminants requires proper assessment of the elevated concentration of copper ion in a wide range of chemical and biological processes in various media such as water, biological, environmental, medical and industrial samples. For practical applications, simple, rapid, reliable, selective and low-cost monitoring systems needed for sensitive determination of both the free metal ions and the complexed metal species of heavy metal ions in natural waters are of potential ecotoxicological concern.

Many methods for the speciation and quantification of Cu^{2+} have been reported: for example, ion selective electrodes [6], anodic stripping voltammetry [7], potentiometric measurement [8], dialysis membranes, ion exchange resins [9], PVC membrane sensor [10], Donnan dialysis [11], charge separation [12], and competitive chelation [13], but the problem of these detection methods includes low sensitivity, high cost and less selectivity. Among the various detection methods available, UV–visible and fluorescence spectroscopy still remain the most frequently used modes for the recognition of physiologically and environmentally important analytes due to their high sensitivity and easy operational use.

In recent years, fluorescent probes for the detection of environmentally and biologically important metal cations have received extensive attention for designing and development of colorimetric or fluorescent chemosensors. Rhodamine dyes belong to the family of xanthene's, owing to their excellent fluorescence properties have also been used as fluorescent chemosensors for detection of ions based on their good photostability, high quantum yield in aqueous solution, low cost, long-wavelength absorption/emission and high molar absorption coefficient. Many unique signaling systems have been developed based on rhodamine which is used

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for selective fluorescent chemodosimeter. Rhodamine-lactams, a group of non-fluorescent rhodamine derivatives, have been extensively explored as fluorogenic and chromogenic sensing platforms for various ions because the spirolactam ring-opening process leads to a turn-on fluorescence change with specific color visible to naked eye on addition of ions [14].

Mercury ion exhibited very good affinity toward sulfure, *e.g.*, rhodamine thiosemicarbazide are reported as colorimeteric sensing for mercury ion [15]; although rhodamine B thiosemicarbazide reported as chromogenic and fluorescent chemosensor for copper ion in aqueous media [16] but on changing solvent system, the same moiety behave as dual sensor for Hg^{2+} as well as Cu^{2+} [17]. A number of Cu^{2+} chemosensors had been developed [18], still there is further need to improve selectivity, sensitivity and efficiency of sensing system for Cu^{2+} with low detection limits, fair solubility of sensor molecules in aqueous solution, easily synthesizable and low cost. Prompted by these findings, we have planned and synthesized rhodamine B semicarbazide **3**, which interestingly showed selectivity only for Cu^{2+} in an aqueous solution, showing detection limits of 1.6×10^{-7} mol/L at neutral pH condition, and proceeded to investigate the applicability of **3** for determination of Cu^{2+} in living cells using HeLa cell lines, which exhibited strong fluorescence as monitored by confocal fluorescence microscopy. The aim of this study was to develop a sensitive and rapid visual as well as fluorescence sensing method to assess copper toxicity in fresh waters as well as in living cells in micro molar concentration level.

2. Experimental

2.1. Substrate and reagents

Rhodamine B, hydrazine carboxamide hydrochloride, phosphorus oxychloride was purchased from Aldrich. Triethyl amine, ethanol, methanol, 1,2-dichloroethane, acetonitrile, water, hexane and ethyl acetate (Samchun chemicals, Korea), chloride and nitrate salts of Fe^{3+}, Fe^{2+}, Cr^{3+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Ag^{2+}, Mn^{2+}, Sr^{2+}, Cs^{2+}, Na^{+}, K^{+}, Li^{+} (Aldrich and Alfa Aesar) were used during experiment.

2.2. Instrumentations

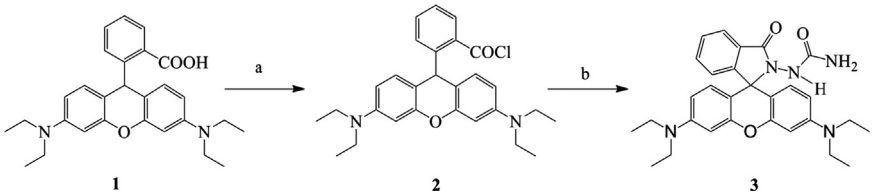
Reaction progress was monitored by thin layer chromatographic (TLC) analysis and R_f values were determined by employing pre-coated silica gel aluminium plates, Kieselgel $60F_{254}$ from Merck (Germany), using *n*-hexane: ethyl acetate, 8:2, as an eluent and TLC was visualized under UV lamp (VL-4. LC, France). Melting points were determined on Fisher scientific (USA) melting point apparatus and are uncorrected. Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}CNMR) spectra were recorded on Varian Inova 400 MHz NMR system (USA) with TMS as an internal standard. Chemical shifts are reported as δ values (ppm) downfield from internal tetramethylsilane of the indicated organic solution. Peak multiplicities are expressed as follows: s, singlet; d, doublet; t, triplet; q, quartet; dt, doublet of triplets and m, multiplet. Mass spectra were recorded on the AB SCIEX Co. 4000 QTRAP® LC/MS/MS System.

2.3. Chemistry

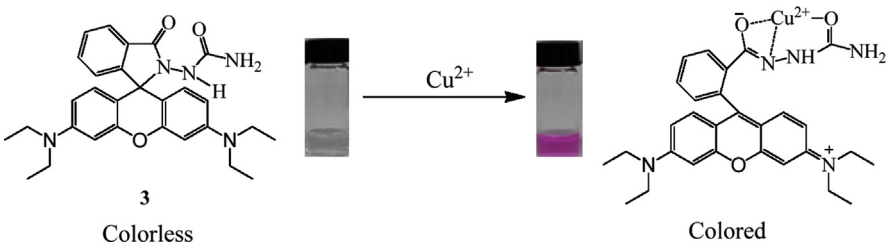
Formation of rhodamine B semicarbazide **3** was indicated by IR spectrum due to disappearance of characteristic broad peak of acid group in the range of 3400–2500 cm^{-1}. The IR spectral data of **3** exhibited characteristic absorption band for primary NH_2 group along with a shoulder at 3306 and 3281 cm^{-1}; while absorption band for the secondary NH group was observed at 3112 cm^{-1}. A strong absorption in the region 1659 and 1638 cm^{-1} was assigned to the carbonyl group of amide linkage. Further confirmation was carried out through ^{1}H NMR spectrum by the disappearance of signals due to hydrogen of carboxylic acid group in rhodamine B **1** at 11 ppm and the appearance of additional signals in the aromatic region in both ^{13}C NMR and ^{1}H NMR spectra (Figs. S1 and S2, Supporting information). Rhodamine B semicarbazide **3** showed strong molecular ion peak $[M+H]^+$ at m/z=500 with maximum intensity in the mass spectrum showing correct mass of **3** (Fig. S4, Supporting information). The reaction pathway adopted for the synthesis of rhodamine B semicarbazide **3** was outlined in Scheme 1.

2.4. Copper chelation mechanism

A number of studies are going on in recent years to understand the ring opening mechanism of rhodamine derivatives [16,17], proposed spirolactam ring opening mechanism of our synthesized probe triggered by copper ion are shown in Scheme 2. In the mass spectrum, a unique peak at m/z 500 corresponding to $[M+H]^+$ was clearly observed when 1 equivalent of Cu^{2+} was added to the methanol solution of **3** (Fig. S4, Supporting information). There was no mass loss occurs in the mass spectrum recorded before and after copper addition, providing an evidence of intramolecular rearrangement upon copper addition in the probe solution.



Scheme 1. Synthesis of probe **3** (rhodamine B semicarbazide): Reagents and conditions. (a) $POCl_3$, $ClCH_2CH_2Cl$, reflux 3 h; (b) hydrazine carboxamide hydrochloride, TEA, MeCN, reflux 5 h.



Scheme 2. Rhodamine B spirolactam ring opening mechanism of probe **3** upon copper chelation in methanol water (1:1, v/v) at pH 7.0.

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2.5. General procedure for the synthesis of rhodamine B semicarbazide 3

Rhodamine B semicarbazide **3** was synthesized according to the reported procedures [19]. Briefly, a solution of rhodamine B (0.45 g, 1 mmol) and 1,2-dichloroethane (12 mL) was stirred and phosphorus oxychloride (0.4 mL) was added drop wise and reaction mixture was heated under reflux for 3 h, then the resulting solution was cooled to room temperature and solvent was removed under reduced pressure to afford rhodamine B acid chloride **2** which was directly used for next step without further purification. Rhodamine B acid chloride **2** was dissolved in acetonitrile (20 mL) and added drop wise into the solution containing hydrazine carboxamide hydrochloride (0.11 g, 1 mmol), TEA (0.5 mL), acetonitrile (20 mL) and allowed to reflux for 5 h, monitored by TLC. After consumption of the starting material, mixture was cooled to room temperature. Evaporation of solvent under reduced pressure left crude rhodamine B semicarbazide **3** as white solid on cooling, which was purified by column chromatography and crystallized on methanol.

2.6. 1-(3′,6′-Bis(diethylamino)-3-oxospiro[isoindoline-1,9′-xanthen]-2-yl)urea (3)

White solid; yield: 58%; mp 210–212 °C; R_f: 0.31 (*n*-hexane: ethyl acetate, 8:2); ^{1}H NMR (400 MHz, CD_3OD) δ 7.93–7.91 (aromatic, 1H, d, J=9 Hz), 7.63–7.57 (aromatic, 2H, dt), 7.15–7.13 (aromatic, 1H, d, J=10.5 Hz), 6.49 (aromatic, 2H, m), 6.41–6.35 (aromatic, 4H, m), 3.39–3.34 (aliphatic, 8H, q, J=17.5), 1.16–1.13 (aliphatic, 12H, t, J=9 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 168.4, 155.6, 150.5, 134.9, 130.9, 129.9, 125.7, 124.2, 109.4, 105.8, 99.1, 68.3, 45.5, 13.0; MS for $C_{29}H_{33}N_5O_3$ (ESI, *m*/*z*), 500 $[M+H]^+$.

3. Results and discussions

A colorimetric/fluorescent probe **3** using rhodamine B semicarbazide have been reported and investigated with highly selective and sensitive recognition toward Cu^{2+} over other examined metal ions (Fe^{3+}, Fe^{2+}, Cr^{3+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Ag^{2+}, Mn^{2+}, Sr^{2+}, Cs^{2+}, Na^+, K^+, Li^+) in aqueous media, in which the spirolactam (non-fluorescent) form converted to the ring-opened (fluorescent) form upon copper addition. The cation-sensing mechanism of probe **3** is based on the change in structure between spirocyclic form to ring opened form triggered by metal cation leads to a spirocycle opening and appearance of pink color as depicted in Scheme 2. The proposed probe was colorless and non-fluorescent in aqueous methanol solution. However, upon addition of Cu^{2+}, the chelation of metal ions with sensor molecules will simultaneously open the spirolactam ring and convert the probe **3** into their ring opened state with dramatic change in color of solution and, remarkably enhanced UV/visible

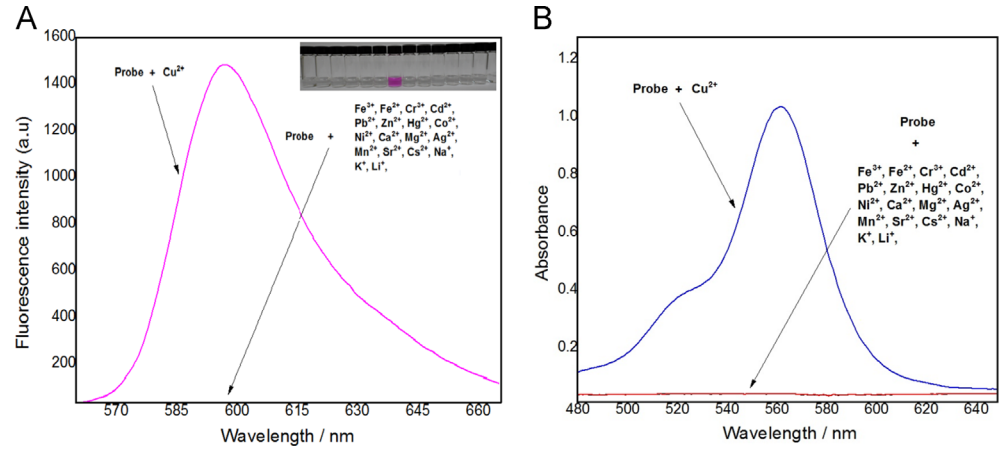

Fig. 1. (A) Emission spectrum of **3** in the presence of Cu^{2+} and various species (Fe^{3+}, Fe^{2+}, Cr^{3+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Ag^{2+}, Mn^{2+}, Sr^{2+}, Cs^{2+}, Na^+, K^+, Li^+) at pH=7.0. Inset photograph showed the visual detection of different metal ions including copper ion (16 μM) by using probe **3** (20 μM) in methanol water (1:1, v/v) at pH 7.0; (B) absorption spectrum of **3** in the presence of Cu^{2+} and various species (Fe^{3+}, Fe^{2+}, Cr^{3+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Ag^{2+}, Mn^{2+}, Sr^{2+}, Cs^{2+}, Na^+, K^+, Li^+) at pH=7.0.

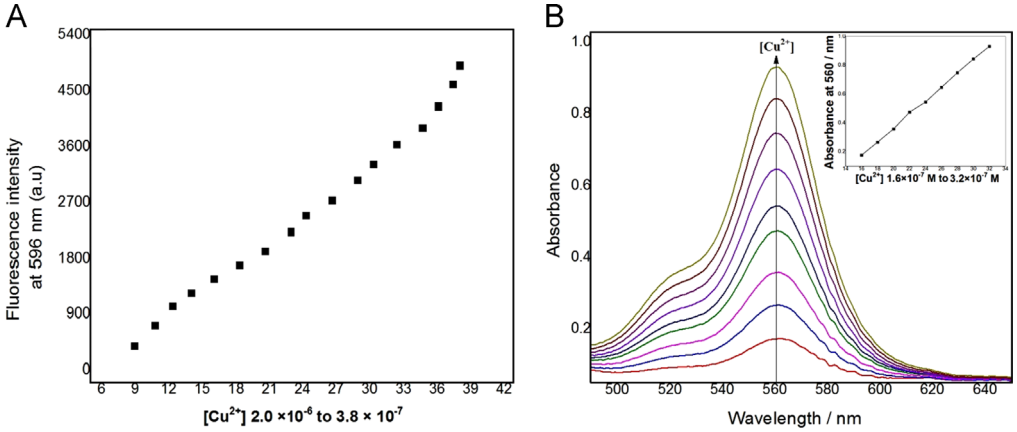

Fig. 2. (A) The fluorescence titration of probe **3** (20 μM) at 596 nm as a function of copper concentration (2–38 μM); (B) absorption spectrum of probe **3** (20 μM) with different concentration of Cu^{2+} (16 μM to 32 μM) in methanol–water (1:1, v/v) at pH 7. The inset shows titration curve by absorbance at 560 nm.

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and fluorescence response showing absorption maxima at 560 nm and emission maxima at 596 nm, behave as significant off/on sensor. In addition, the limit of detection for Cu^{2+} in aqueous methanol (1:1, v/v) was found to be 1.6×10^{-7} mol/L at neutral pH.

3.1. Fluorescence and UV–visible spectra of probe with copper ion and competitive ions

Selectivity behavior of sensing material dependent on the structure and nature of the sensing element used in its composition, our synthesized probe showed good interactions with copper ions. The results revealed that probe has a selective tendency towards Cu^{2+} ions in comparison to other tested cations, meanwhile, common metal ions showed negligible detection interference except Cu^{2+} with no fluorescence emission as well as no absorption response (Fig. 1).

3.2. Fluorescence and UV–visible titration of probe against different concentration of Cu^{2+}

Concentration of Cu^{2+} showed prominent effect on the emission and absorption intensity of probe **3**, accompanying the increase of the concentrations of Cu^{2+}, the peak intensity of the maximum emission at 596 nm and maximum absorption at 560 nm increased gradually, suggesting the formation of the Cu^{2+} induced ring opening of the spirolactam form as shown in Fig. 2.

3.3. pH effect on the probe sensing mechanism

The influence of different acid concentration on response mechanism of probe **3** was evaluated. The results showed that relative fluorescence intensity or spectroscopic properties of probe **3** strongly dependent on pH of solution investigated in the pH range of 2–10 as shown in Fig. 3. The titration curve of free chemosensor in MeOH/Tris–HCl buffer did not show obvious characteristic color at neutral pH, suggesting that spirolactam tautomer of **3** was insensitive at the neutral pH, while acidic pH gives pink color solution due to spirolactam ring opening of **3** triggered by activation of carbonyl group of spirolactam ring by protonation due to acids. However, the addition of Cu^{2+} at neutral pH led to the fluorescence enhancement, which is attributed to the Cu^{2+} activated spirolactam ring opening of the probe **3**.

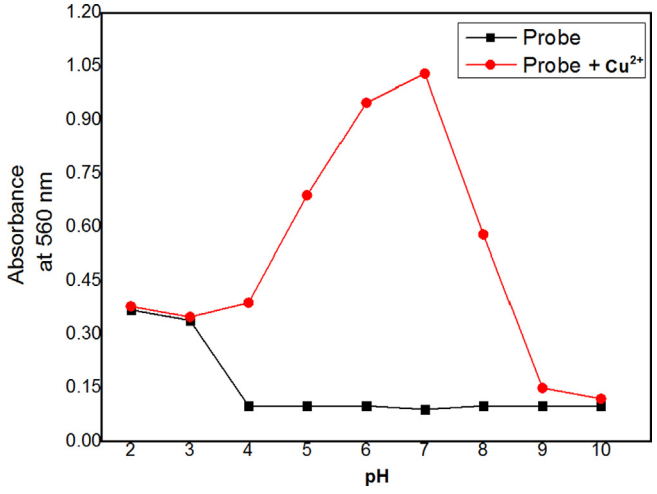

Fig. 3. Absorption intensity variation with alteration of pH from 2 to 10, in the presence of MeOH/Tris–HCl buffer solution with different pH conditions.

3.4. Effect of media on response mechanism of probe

The effect of water content on the UV–visible spectral measurement of Cu^{2+} was investigated, as shown in Fig. 4, it can be observed that the sensor **3** exhibits sensitive response to Cu^{2+} in aqueous methanol, (1:1, v/v) solvent system, as biological molecules are soluble in aqueous media, so, the synthesized sensing system can equally be applicable for copper sensing in biological systems as

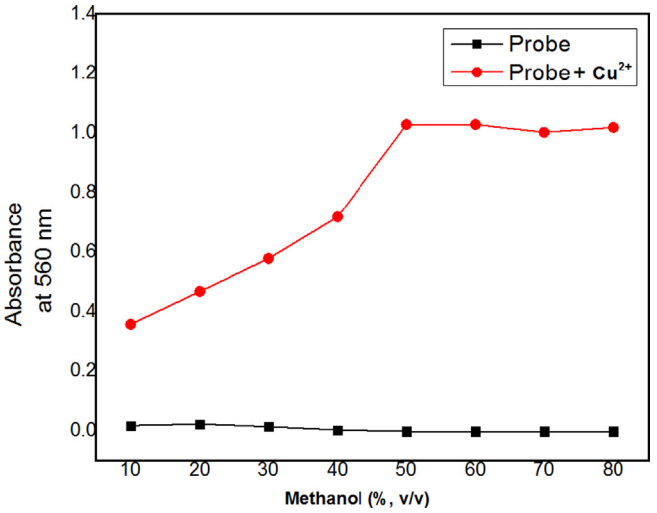

Fig. 4. Variation of absorption intensity of probe **3** (20 μM) with and without copper ion (16 μM) at 560 nm upon different water: methanol, ratio; while base line signal are due to only probe without metal addition in aqueous methanol solvent system.

Table 1
Solvent effect on the copper chelation with probe.

Solvent	Absorption intensity
Water	0.08
Methanol:water (1:1, v/v)	1.03
Ethanol:water (1:1, v/v)	0.75
Acetonitrile:water (1:1, v/v)	1.02
Chloroform:water (1:1, v/v)	0.03

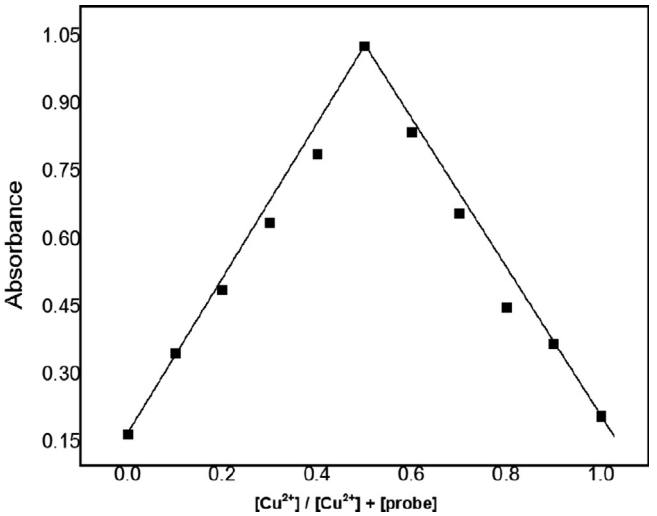

Fig. 5. Job's plot of probe copper complex in MeOH/Tris–HCl buffer solution give rise to 1:1 stoichiometry for the complexation reaction. Total concentration of probe+copper ion was kept same as 100 μM.

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well. The absorption signal intensity gradually increased and reached at maximum value at 50% aqueous methanol solvent system and signal intensity become stable above 50% aqueous methanol solution. Therefore, 50% aqueous methanol media was selected for the fluorescence excitation, emission and UV–visible absorption spectral recording while probe alone showed no absorption signal at 560 nm with used solvent system at neutral pH condition.

The coordination reaction was greatly dependent on the nature of solvent; organic solvent exert significant effect on the absorption and emission intensity, maximum absorption response was observed in mixed methanol water solution (1:1, v/v) and minimum response obtained in case of chloroform water solution. The effect of different aqueous organic solvent on the absorption intensity upon chelation of copper ion with probe is tabulated in Table 1.

3.5. Job's plot for stoichiometric calculation

The stoichiometry of probe copper complex was determined by the Job's method for absorbance measurement [20]. Maximum absorption intensity was observed when the molecular fraction of Cu^{2+} was 0.5, indicating 1:1 stoichiometric complex formation (Fig. 5).

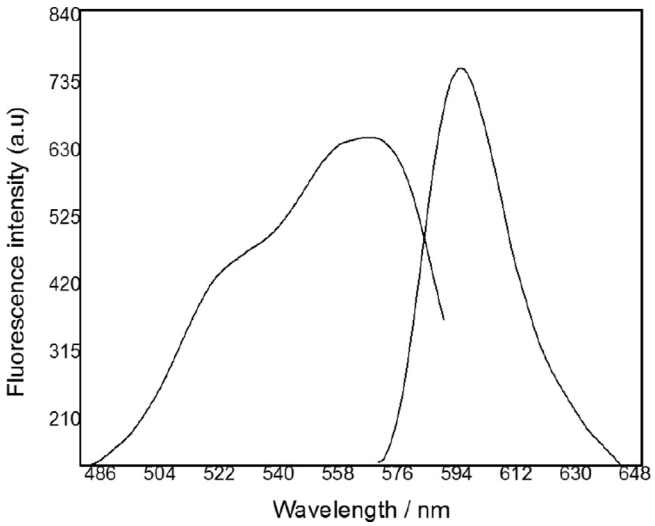

Fig. 6. Fluorescence excitation and emission spectrum of **3** in the presence of Cu^{2+} in aqueous methanol (1:1, v/v) at pH 7.0.

3.6. Stokes shift calculation

The difference between positions of band maxima of excitation and emission spectra of the same electronic transition is called Stokes shift, which comes to be 1078 cm^{-1} for our reaction solution (probe+Cu^{2+}) in aqueous methanol, calculated by Eq. (1), [21]. Probe **3** showed significant Stokes shift for easy separation of excitation and emission signal (Fig. 6).

$$(\upsilon A - \upsilon F) = \left(\frac{1}{\lambda A} - \frac{1}{\lambda F}\right) \times 10^7 \quad (1)$$

where υA and υF are the absorption and fluorescence frequencies, λA and λF are the absorption maxima and fluorescence emission maxima, respectively.

3.7. Fluorescence quantum yield determination

Probe **3** exhibited fluorescence quantum yield of $\Phi_{FL}=0.44$ (relative to the standard rhodamine B in methanol, $\Phi_{std}=0.65$) [22], calculated by using Eq. (2), [23].

$$\Phi_{unk} = \Phi_{std}(I_{unk}/A_{unk})(A_{std}/I_{std})(\eta_{unk}/\eta_{std})^2 \quad (2)$$

where Φ_{unk} is the fluorescence quantum yield of the sample **3**, Φ_{std} is the quantum yield of the standard, I_{unk} and I_{std} are the integrated fluorescence intensities of the sample and the standard, respectively, A_{unk} and A_{std} are the absorbance's of sample and the standard at the absorption wavelength, respectively, η_{unk} and η_{std} are the refractive indices of corresponding solutions.

3.8. Imaging of HeLa cells incubated with Cu^{2+} and 3

To investigate the applicability of synthesized sensing system for Cu^{2+} in living cells, confocal fluorescence spectroscopic studies were conducted by incubating HeLa cells with probe for 20 h and $CuCl_2$ was stained in growth media for 1 h. Emission was collected by the green channel at 530 ± 10 nm and the red channel at 590 ± 25. Briefly, incubation of HeLa cells with probe (200 μM) and HeLa cells with $CuCl_2$ (20 μM) exhibited no intracellular fluorescence (images a and b, Fig. 7); while incubation of HeLa

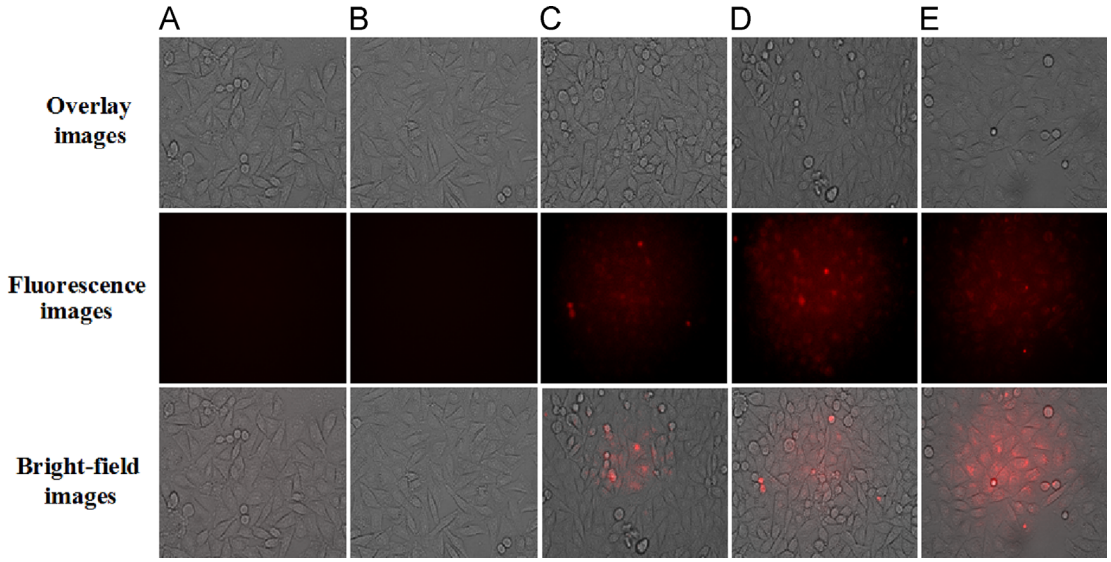

Fig. 7. Confocal fluorescence microscopic images; (a) HeLa cells+probe (200 μM), (b) HeLa cells+$CuCl_2$ (20 μM), (c) HeLa cells+probe (50 μM)+$CuCl_2$ (5 μM), (d) HeLa cells+probe (100 μM)+$CuCl_2$ (10 μM), (e) HeLa cells+probe (200 μM)+$CuCl_2$ (20 μM).

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cells, probe and $CuCl_2$ with varying concentration generated remarkable intracellular fluorescence (images c, d and e, Fig. 7). The results from the confocal fluorescence microscopic analysis of treated cells showed that probe **3** can be used for monitoring Cu^{2+} within biological samples.

4. Conclusion

In conclusion, this study has been demonstrated for visual detection of copper ion in aqueous solution and has provided a simple complexation model for quantitative measurements with high accuracy. Confocal fluorescence microscopic images were obtained by incubating probe **3** and Cu^{2+} with HeLa cells. Absorption and emission spectra of probe were recorded by using copper ion with competitive ions (Fe^{3+}, Fe^{2+}, Cr^{3+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Ag^{2+}, Mn^{2+}, Sr^{2+}, Cs^{2+}, Na^{+}, K^{+}, Li^{+}) in aqueous methanol (1:1, v/v) at pH=7.0. Synthesized probe exhibited high selectivity for copper ion showing strong absorption at 560 nm and emission at 596 nm, while there was no absorption and emission peak in the range of 500–650 nm by incubating probe solution with other inorganic metallic ions; proved an "off/on" fluorescence and colorimetric sensor for the selective signaling of Cu^{2+}. The probe switches to a highly fluorescent complex upon Cu^{2+} chelation under physiological conditions.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jlumin.2013.08.044.

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